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ATTORNEY DOCKET NO. 21085.0136U1

Page 1

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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040605

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. § 1.53(c).

Docket Number		21085.0136U1		Type a Plus Sign (+) inside this box	+
INVENTOR(s)					
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and Either State or Foreign Country)		
KAPPES	John	C.	5284 Birdsong Rd., Birmingham, AL 35242		
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TITLE OF INVENTION (500 characters max)					
AMINO ACID RESIDUES IN THE HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 REVERSE TRANSCRIPTASE TRYPTOPHAN-REPEAT MOTIF REQUIRED FOR SUBUNIT INTERACTION <i>IN VIVO</i>					
CORRESPONDENCE ADDRESS					
Customer Number 23859					
ENCLOSED APPLICATION PARTS (Check All That Apply)					
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<p>Application Size Fee: If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$250 (\$125 for small entity) for each additional 50 sheets or fraction thereof. See 35 USC § 41(a)(1)(G) and 37 CFR § 1.16(s).</p>					

METHOD PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (Check One)

- ☒ Applicant claims small entity status. See 37 CFR § 1.27.
- ☒ A Credit Card Payment Form PTO-2038 is enclosed to cover the filing fees.
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- ☐ The Commissioner is hereby authorized to charge filing fees and the application size fee (if applicable) or credit any overpayment to Deposit Account Number _____.
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FILING FEE AMOUNT

\$100.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

- ☐ No.
- ☒ Yes. The name of the U.S. Government agency and the Government contract number are:
National Institutes of Health (AI 47714; P30-AI27767; R24 DK-64400) and Department of Veterans Affairs

Respectfully submitted,

Signature Tina W. McKeon

Date 4/6/05

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Registration No.: 43,791

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)	
)	
Kappes et al.)	Art Unit: Unassigned
)	
Application No.: Unassigned)	Examiner: Unassigned
)	
Filed: Concurrently)	Confirmation No. Unassigned
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FOR: "AMINO ACID RESIDUES IN THE HUMAN)	
IMMUNODEFICIENCY VIRUS TYPE-1)	
REVERSE TRANSCRIPTASE TRYPTOPHAN-)	
REPEAT MOTIF REQUIRED FOR SUBUNIT)	
INTERACTION <i>IN VIVO</i> ")	

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April 6, 2005

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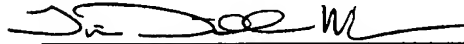
Pursuant to 37 C.F.R. § 1.136(a)(3), the Commissioner is hereby requested and authorized to treat any concurrent or future reply in the above-identified application, requiring a petition for an extension of time for its timely submission, as incorporating a petition for extension of time for the appropriate length of time.

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PATENT**

The Commissioner is hereby authorized to charge any fees which may be required, or
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Respectfully submitted,

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UTILITY PATENT - PROVISIONAL FILING**

PROVISIONAL APPLICATION FOR LETTERS PATENT

TO ALL WHOM IT MAY CONCERN:

**Be it known that we, John C. KAPPES and Alok MULKY, residing respectively at
5284 Birdsong Rd., Birmingham, AL 35242 and 1850 Arboretum Circle, Birmingham, AL
35216 have invented new and useful improvements in**

**AMINO ACID RESIDUES IN THE HUMAN IMMUNODEFICIENCY VIRUS TYPE-1
REVERSE TRANSCRIPTASE TRYPTOPHAN-REPEAT MOTIF REQUIRED FOR
SUBUNIT INTERACTION IN VIVO**

for which the following is a specification.

Abstract

The human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) functions as a heterodimer (p51/p66), which makes disruption of subunit interactions a possible target for antiviral drug design. Our understanding of subunit interface interactions has been limited by the lack of virus-based approaches for studying the heterodimer. Therefore, we developed a novel subunit-specific mutagenesis approach that enables precise molecular analysis of the heterodimer in the context of infectious HIV-1 particles. In this study, we analyzed the contributions of amino acids comprising the Trp-motif to RT subunit interaction and function. Our results reveal important inter- and intra-subunit interactions of residues in the Trp-motif. A tryptophan cluster in p51 (W398, W402, W406, W414), proximal to the interface, was found to be important for p51/p66 interaction and stability. At the dimer interface, residues W401, Y405 and N363 in p51 and W410 in p66 mediate inter-subunit interactions. The W401 residue is critical for RT dimerization, exerting distinct effects in p51 and p66. Our analysis of the RT heterodimerization enhancing non-nucleoside RT inhibitor (NNRTI), efavirenz, indicates that the effects of drugs on RT dimer stability can be examined in human cells. Thus, this report provides the first description of subunit-specific molecular interactions that affect RT heterodimer function and virus infection *in vivo*. Moreover, with heightened interest in novel RT inhibitors that affect dimerization, we demonstrate the ability to assess the effects of RT inhibitors on subunit interactions in a physiologically relevant context.

Introduction

The reverse transcriptase (RT) of human immunodeficiency virus type-1 (HIV-1) is a heterodimeric enzyme comprised of 51-kDa and 66-kDa subunits ^{1; 2}. The RT heterodimer (p51/p66) is generated via proteolytic processing of the Gag-Pol polyprotein precursor (Pr160^{Gag-Pol}) by the viral protease (PR) during assembly/maturation of the virus particle (see ^{3; 4} for review). The p66 subunit is divided into two domains, polymerase and RNase H. The p51 subunit is produced by PR cleavage of the p66 subunit and removal of the RNase H domain ^{5; 6}. Crystallographic analysis subdivides the polymerase domain into the fingers, palm, thumb and connection subdomains ⁷. Since the subunits are derived from the same coding region, and the relative arrangement of RT subdomains differs markedly between p51 and p66, a mutation in one subunit is structurally and functionally non-equivalent to the same mutation in the other subunit. For this reason, and because mutations in the RT domain of Pr160^{Gag-Pol} may be pleiotropic, impairing virus assembly or maturation ^{8; 9; 10}, subunit-specific structure/function studies of RT have not been possible with infectious HIV-1.

HIV-1 RT dimerization has been investigated using a number of complementary non-physiologic, in vitro approaches. Formation of the heterodimer has been defined as a two-step process ^{11; 12}. The first step involves a concentration-dependent association of the two subunits. This is followed by a slow isomerization/maturation step that involves interactions of the fingers and thumb of p51 with the palm and RNase H of p66, respectively. Specifically, residues 52–55 and 135–140 of the p51 fingers appear to interact with residues 85–96 of the p66 palm ^{13; 14; 15}.

while residues 280–295 of the p51 thumb subdomain appear to contact residues 536–545 of RNase H^{16; 17; 18; 19}. The first step in RT dimerization apparently involves interactions between hydrophobic residues in the connection subdomains of p51 and p66. This is believed to include residues W401–W410 of p66 and residues P392–W401 of p51^{15; 20; 21}. The connection subdomain is distinctive in having six tryptophans and a tyrosine between amino acids 398–414. This motif is well conserved among the primate lentiviruses, and has been appropriately dubbed the tryptophan-repeat motif (Trp-motif). In a recent study, using a yeast two-hybrid approach to analyze Trp-motif mutations, residues p66^{W401} and p66^{W414} were shown to be involved in RT dimerization²¹. Mutagenesis of other aromatic amino acids that lie between these two residues did not affect subunit interaction. Since p66^{W401} and p66^{W414} are not located at the dimer interface, the authors suggested that repositioning of structural elements between these residues accounted for their results. A comprehensive investigation of the Trp-motif has not been possible due to an inability to express most recombinant p51 Trp-motif mutants in yeast and bacteria²¹. Others have also reported that mutation of certain hydrophobic residues impairs recombinant p51 protein expression in bacteria, while the same mutation in p66 is expressed normally¹⁰.

The HIV-1 RT functions as a heterodimer and several studies have suggested that the dimerization process of RT could be targeted for HIV antiretroviral therapy^{22; 23}. Synthetic peptides corresponding to the connection subdomain (Trp-motif) have been reported to disrupt dimerization^{20; 23; 24}. Perhaps most notably, a short peptide matching RT residues 395–404 was shown to inhibit heterodimerization in vitro and virus replication in cell culture²⁰. Recent studies of nonnucleoside reverse transcriptase inhibitors (NNRTI) have heightened interest in compounds that interfere with RT conformational flexibility as a novel drug design concept^{25; 26}.

NNRTI are a group of small hydrophobic compounds with diverse structures that inhibit HIV-1 RT (see ²⁷ for review). NNRTIs interact with HIV-1 RT by binding to a site on the p66 subunit of the heterodimer. This results in both short-range and long-range distortions of the RT structure. NNRTIs have been shown to interfere directly with the global hinge-bending mechanism that controls the cooperative motions of the p66 fingers and thumb subdomains required for RT function ^{28, 29}. In yeast, several NNRTIs were recently shown to enhance p51/p66 subunit association as a result of a specific interaction of drug with p66 ³⁰. Clearly, understanding the biophysical parameters defining subunit interactions will be integral to designing effective inhibitors of RT.

We recently reported a novel approach that enables subunit-specific analysis of p51 and p66 in infectious virions ³¹. We demonstrated that a Vpr-p51 fusion protein is incorporated into HIV-1 virions through an interaction between Vpr and the Gag precursor polyprotein, and when coexpressed p66 is specifically and selectively packaged as a Vpr-p51/p66 complex. Processing by the viral PR liberates Vpr, generating functional heterodimeric RT (p51/p66) and infectious viral particles. By exploiting this approach we have now determined the contribution of amino acid residues comprising the Trp-motif to RT subunit interaction and virus infection. We demonstrate that inter-subunit interactions between the connection subdomains include W401, Y405 and N363 in p51 and W410 in p66, and that mutation of these residues impairs RT function and virus infectivity. The W401 residue of the Trp-motif was found to be of central importance. Mutation of this amino acid simultaneously in both subunits is deleterious to RT dimerization and virus infection. The RT heterodimerization enhancing drug, efavirenz (EFV), rescued this dimerization defect in a dose-dependent manner. Additionally, we demonstrate that

intra-subunit interactions between tryptophans comprising a hydrophobic cluster (W398, W402, W406, W414) proximal to the connection subdomain interface are important for p51/p66 subunit interaction and stability. Our findings reveal important new insights into the biophysical nature of the Trp-motif and its role at the connection subdomain dimer interface.

Results

Structural analysis of the putative RT dimerization domain (tryptophan motif)

The Trp-motif of HIV-1 is comprised of aromatic amino acids in the connection subdomain (between amino acid positions 398 and 414). Alignment analysis shows that the six tryptophans (W398, W401, W402, W406, W410 and W414) and tyrosine (Y405) residues are conserved within the connection subdomain of most primate lentiviruses (Figure 1A). The most conserved residue amongst all the lentiviruses is W398 (data not shown). To understand the interactions at the dimerization interface between the two connection subdomains of HIV-1 RT, we compared several crystal structures of HIV-1 RT, including unliganded (1DLO), in complex with substrates (pdb codes 1TO5, 1RTD, 1HYS, 1N6Q) or NNRTIs (1HNI, 1SV5, 1S6P, 1S9E, 1DTT, 1BMQ, 1FK9). The overall structure of the dimerization interface appears to be conserved among the various RT complexes. Stabilization of the heterodimer appears to involve direct, as well as indirect interactions between residues from the two subunits. Specifically, a key direct interaction appears to involve three p51 residues from the β 18- α K (N363) loop, the α L helix (W401), and the α L- β 20 loop (Y405), that are within interacting distance of residue W410.

located in the α L- β 20 loop of p66 (Figures 1B, 1C, and 1D). In addition to these interactions, the W401 in p51 is also within interacting distance with p66 residue P412 at the base of the β 20-sheet in p66. Indirect interactions may also play a role and involve residues that are proximal to the p66 or the p51 part of the interface. In the p51 subunit, a cluster of four Trp residues (W398, W402, W406 and W414) is proximal to the p51 interface residues (Y405, N363, and W401) (Figures 1B, 1C, and 1D).

Expression and virion incorporation of heteromeric RT containing p51 Trp-motif mutations

The p51 Trp-motif residues were independently mutated to alanines in the pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) expression plasmid. Wildtype and each of the mutant DNAs were cotransfected into 293T cells with the RT-IN defective M7 proviral DNA, and progeny virions were examined by immunoblot analysis. The control RT-IN-minus M7 particles (Figure 2A, lane 2) did not contain RT. A similar level of both RT subunits (p51 and p66) was detected in particles derived by cotransfection of M7 and the wildtype *vpr-p51/p66* expression plasmid (lane 3). The pLR2P-vpr- Δ p51-IRES-p66 (*vpr- Δ p51/p66*) control plasmid does not express p51, and the absence of detectable p66 (lane 4) confirmed that its incorporation was dependent on the expression of p51 (Vpr-p51). Analysis of p51^{W398A}/p66 (lane 5) showed p51 and p66 in the virion, however, an additional band was also detected migrating just below p66. This band was confirmed to be a product of the p66 subunit by probing with mAb (7E5) specific to the RNase H domain (data not shown). To a lesser extent, a similar p66 product was also seen in some of the other p51- mutants. Notably, the aberrant p66 product seemed to associate with mutants of

residues (W398A, W402A, W406A and W414A) that cluster together proximal to the heterodimer interface (Figure 1C). Wildtype HIV-1 SG3 virions were analyzed as an additional control (lane 1). Immunoblot analysis using mAb to CA confirmed that approximately the same amount of each virus was analyzed (Figure 2B). Examination of transfected cells by immunoblotting with polyclonal anti-Vpr serum (Figure 2C) demonstrated that all of the mutants expressed Vpr-p51 (lanes 5-11) at levels similar to that of wildtype Vpr-p51 (lane 3). A replica blot probed with 7E5 mAb confirmed that p66 was expressed at a similar level among the transfected cells (Figure 2D). The amount of cellular protein analyzed was similar, as demonstrated by probing for the α -tubulin protein (Figure 2E).

To examine if the aberrant p66 was due to misprocessing by the viral protease (PR), 293T cells were cotransfected with a PR-defective proviral DNA (PR catalytic mutant: D25A) and *vpr-p51^{W398A}/p66*. Detection of the aberrant p66 product in these virions (data not shown), suggested that it was generated independently of the HIV-1 PR.

Functional analysis of p51 Trp-motif RT mutants

The functionality of the p51 Trp-motif mutants was analyzed in a single-round infectivity assay, using the TZM-bl reporter cell line³³. Virions were generated by cotransfecting 293T cells with M7, wildtype or mutant *vpr-p51/p66* and *vpr-IN*. *vpr-IN* was included since M7 lacks IN, which is required for efficient initiation of reverse transcription and for integration of the nascent viral cDNA^{31; 39}. The infectivity of virions containing the wildtype *trans*-RT (Vpr-p51/p66) was normalized to 100% (Figure 2F, lane 1). The infectivity of M7 derived by cotransfection with *vpr- Δ p51/p66* was less than 0.2% compared to *vpr-p51/p66* (lane 2). Mutations in the tryptophan

cluster (W398A, W402A, W406A and W414A) decreased infectivity to less than 50% (lanes 3, 5, 7 and 9, respectively), with the W398A mutant being the most defective. The infectivity of the p51^{W401A}/p66 mutant (lane 4) was similar to that of wildtype, while the p51^{Y405A}/p66 mutant (lane 6) was reduced to about 50%. The p51^{W410A}/p66 mutant (lane 8) had little effect on infectivity, consistent with our previous report on this mutant ³¹.

Subunit-specific mutagenesis of Trp-motif residues at the heterodimer interface

Our analysis of inter-subunit interactions was focused initially on mutagenesis of individual residues to either alanine or leucine. The infectivity of the wildtype RT *trans*-heterodimer was normalized to 100% (Figure 3A, lane 1). The *vpr-Δp51/p66* was less than 0.2% infectious (lane 2), as reported previously. Replacement of p51^{W401} with either alanine or leucine did not affect viral infectivity (lanes 3 and 4). Both the p51^{Y405A}/p66 and p51^{Y405L}/p66 mutant RTs reduced infectivity to approximately half of that of the wildtype *trans*-heterodimer (lanes 5 and 6, respectively). Mutation of N363 in p51 to alanine also reduced infectivity, albeit to a lesser extent than the 405 mutations (lane 7). The replacement of p66^{W410} with alanine caused a slight reduction in infectivity (lane 8), while the leucine substitution had no effect (lane 9). The p51/p66^{L234A} and p51/p66^{W401A} mutants, reported previously as mutations that affect dimer formation, were included as controls in our experiments ^{21; 40}. The p51/p66^{L234A} mutant reduced infectivity to less than 5% (lane 10), while the p51/p66^{W401A} mutant was approximately 40% infectious (lane 11).

To further delineate these Trp-motif interactions, residues that lie within interacting distance of each other were mutated in pairs. Mutations were made in conjunction at residues.

W401 and W410 of p51 and p66, respectively. Infectivity analysis of p51^{W401A}/p66^{W410A}, p51^{W401A}/p66^{W410L} and p51^{W401L}/p66^{W410A} suggested that mutagenesis of both residues together reduced viral infectivity (approximately 40%) to a significantly greater extent compared to that of the single mutations (Figure 3B, lanes 1, 2 and 3). Analysis of RT containing simultaneous mutations at p51^{Y405} and p66^{W410} indicated that substitution of both residues with alanine decreased infectivity to about 25% of the wildtype heterodimer (lane 4). In contrast, the infectivity of the p51^{Y405A}/p66^{W410L} double mutant (lane 5) was comparable to that of the p51^{Y405A}/p66 single mutant, suggesting that mutagenesis of p66^{W410} to leucine does not affect its interaction with Y405 of p51. Our model predicts that the residue N363 in p51 interacts with both p51^{Y405} and p66^{W410}. The p51^{N363A;Y405A}/p66 (lane 6) and p51^{N363A}/p66^{W410A} (lane 7) virions had similar infectivity, which was reduced to approximately 35% of wildtype and substantially lower than the respective single mutants. Immunoblot analysis detected only a slight reduction in Vpr-p51-mediated p66 incorporation in some of the double mutants (data not shown).

Analysis of the inter-subunit interface in provirus

Careful consideration of our results indicated an interaction at the dimer interface between residues p51^{W401} and p66^{W410} that is important for subunit interaction. Additional analysis of the W401A and W410A mutations was conducted in the context of the complete HIV-1 NL4-3 proviral clone. The wildtype or mutant proviral DNAs were transfected into 293T cells and progeny virions were analyzed for infectivity. The infectivity of virus containing the W401A mutation was less than 5% of that of wildtype (Figure 4A, lanes 1 and 3). In contrast, W410A caused an increase in virus infectivity (lane 4). The non-infectious RT-minus M7 clone

was included as negative control (lane 2). Notably, immunoblot analysis showed a significantly reduced amount of the W401 mutant RT in virions, compared to either the wildtype or W410A mutant (Figure 4B). Probing a replica blot with mAb to CA confirmed that approximately the same amount of each virus was analyzed (Figure 4C).

Analysis of W401A mutation in the RT trans-heterodimer

To determine the effect of the W401A proviral DNA mutation on RT, the mutation was analyzed by subunit-specific *trans*-complementation, wherein the mutation was present p51, p66 or both p51 and p66. The infectivity of virions complemented with the wildtype *trans*-heterodimeric RT was normalized to 100% (Figure 5A, lane 1). Subunit-specific mutagenesis of p51 (p51^{W401A}/p66) did not significantly affect viral infectivity, as described above (lane 3), while mutagenesis of the p66 subunit (p51/p66^{W401A}) reduced infectivity to about 40% (lane 4). The effect of this mutation in both subunits (p51^{W401A}/p66^{W401A}) was quite dramatic (lane 5), reducing infectivity to levels similar to that observed for the W401A mutant provirus.

Analysis of virions produced by coexpressing *vpr-p51^{W401A}/p66* demonstrated, as expected, wildtype levels of both subunits (Figure 5B, lanes 1 and 3). However, when the W401A mutation was present in the p66 subunit (*vpr-p51/p66^{W401A}*), the incorporation of p66 into M7 virions was reduced (lane 4). Interestingly, the presence of W401A in both p51 and p66 further reduced the amount of p66 detected in virions (lane 5). In all cases, reduced virion p66 was also seen using a polyclonal RT antiserum and the amount of p66 expressed in the cells was normal (data not shown). The decrease in virion p66 observed with the p51/p66^{W401A} and p51^{W401A}/p66^{W401A} mutants was identical to that observed when virions were produced using a

PR-defective proviral DNA (PR catalytic mutant: D25A) in place of M7 (data not shown). This result confirmed that less p66 was detected in the M7 virions because the W401A mutation(s) impaired p66 virion incorporation. Importantly, this result ruled out the possibility that less p66 was detected due to overprocessing and conversion of p66 to p51 by the viral protease subsequent to virion assembly.

To analyze the infectivity of *trans*-RT complemented virions in a target cells that is more physiologically relevant, the JLTRG-R5 reporter cell line was used. These cells are derived from JLTRG cells and are of Jurkat T cell lineage^{34, 41}. The JLTRG-R5 cells have stably integrated EGFP reporter under control of the HIV-1 LTR, and thus EGFP expression is induced by virus infection. Infectivity for the wildtype *trans*-RT heterodimer was normalized to 100% (Figure 5C, lane 1). The Δ p51/p66 exhibited infectivity below 5% (lane 2). The W401A mutation in p51 did not affect viral infectivity (lane 3), while W401A in p66 reduced infectivity to about 50% of that of the wildtype *trans*-RT (lane 4). The presence of W401A simultaneously in p51 and p66 significantly decreased infectivity (lane 5). These results were consistent with those generated using the TZM-bl assay, which was used for analysis in a parallel experiment. The ability to analyze virions containing *trans*-heteromeric RT using a T cell line emphasizes the biological relevance of our approach. The results indicate that the *trans*-RT heterodimer complemented virions can be analyzed in multiple human-derived reporter cell lines including more physiologically relevant T cell lines.

Efavirenz enhances subunit interaction in the trans-RT W401A double mutant

To examine the effect of dimerization enhancing drugs on the dimerization-defective W401A mutant *trans*-heterodimeric RT, EFV was added to the producer cells (transfected 293T cells) at concentrations ranging from 0.01-1.0 μ M. Examination of virion-associated p66^{W401A} incorporation, which is dependent upon interaction with p51^{W401A} (Vpr-p51^{W401A}), showed that EFV rescued subunit dimerization in a dose-dependent manner (Figure 6A). Equal virion protein loading was confirmed by probing a replica blot with anti-CA mAb (Figure 6B). The amount of virion-associated p51 and Vpr-p51 was equal in both the absence and presence of drug (data not shown). Similar results were also observed for other second generation NNRTIs that show promise in early clinical trials (data not shown).

Discussion

RT subunit interactions have been extensively analyzed using biochemical and other non-physiological methods. The lack of a virus-based approach for analyzing subunit interactions has hampered our understanding of the dimerization process, and possibly the development of RT antagonists that affect subunit interactions. Two significant problems have hindered structure/function studies of RT using infectious virus. First, RT is encoded and assembled into virions as part of the Pr160^{Gag-Pol} polyprotein, and consequently, mutations in RT/Pr160^{Gag-Pol} may be pleiotropic, affecting multiple steps of the viral life cycle such as assembly, maturation, etc.^{8; 9; 10; 42}. Analogous with our results for the W401A proviral mutant, Yu *et al.* have reported that mutations in the polymerase primer grip decrease virion-associated RT due in part to

premature Gag-Pol processing⁸. The second problem is due to the heterodimeric nature of the RT. The asymmetry of the p51 and p66 subdomains entails that a mutation in one subunit is structurally and functionally non-equivalent to the same mutation in the other subunit. Thus, we developed a novel *trans*-complementation approach for analyzing the RT heterodimer in precise molecular detail in the context of infectious virions. By exploiting this approach, we have addressed several relevant questions concerning HIV-1 RT biology that were previously experimentally inaccessible. Primarily, these include (i) the role of hydrophobic amino acid residues comprising the Trp-motif for subunit interaction and RT function, (ii) the contribution of amino acids at the p51/p66 connection subdomain interface to RT dimerization and virus infection, and (iii) the availability of a biologic approach capable of assessing the effects of both dimerization enhancing and disrupting drugs.

Several studies have suggested that the hydrophobic residues in the Trp-motif are important for RT dimerization^{15; 43}. A recent study, utilizing a yeast-two hybrid approach demonstrated that residues p66^{W401} and p66^{W414} are important for subunit interaction²¹. Because neither of these residues is located at the dimer interface, the authors inferred that repositioning of structural elements in the p66 α L- β 20 loop, which lie between these residues, affected p51-p66 interaction and accounted for their observation. Since heterodimer formation was not affected by mutagenesis of the other aromatic amino acids in the p66 α L- β 20 loop, it was suggested that backbone carbonyl groups in the loop and not specific amino acid side-chains provide the important interactions at this interface. However, the inability to express most of the p51 Trp-motif mutants precluded a comprehensive examination of this p51/p66 interface. Structural analysis of interactions in the Trp-motif with residues at the subunit interface in...

several complexes of RT with substrate or inhibitors shows that the side-chain of W410 in the p66 α L- β 20 loop is consistently within interacting distance of p51 residues W401, Y405, and N363 (Figure 1D). *trans*-complementation analysis of these putative inter-subunit interactions showed that mutation of individual residues at this interface, with the exception of p51^{W401}, caused a measurable decrease in virus infectivity. Simultaneous mutagenesis of two inter-subunit residues within interacting distance of each other further impaired viral infectivity, suggesting that this effect was most likely due to effects on subunit interactions. The data from immunoblot (not shown) indicate similar to wildtype (Vpr-p51/p66) levels and processing of the mutant *trans*-RT of the two subunits. The decreased infectivity could be explained as follows: (i) The destabilization of the heterodimer is not sufficient to cause disassociation. Thermodynamic evaluations of p51/p66 have estimated a relatively high Gibbs free energy of dimer stabilization (approximately 10–12 kcal/mol), corresponding to a dissociation constant of ~ 10.0 nM^{14,44}. This has necessitated that *in vitro* dimer dissociation studies be carried out in the presence of denaturants like urea or acetonitrile^{14, 23, 45}. Thus, a precedent for dimer destabilization without disassociation in the absence of extrinsic factors exists. However, destabilization, albeit not sufficient to cause disassociation, could impair RT function and consequently virus infectivity. (ii) It is possible that mutations may have global effects on the folding of the subunits.

The most severe effect on heterodimerization was observed for the p51^{W401A}/p66^{W401A} mutant. The presence of W401A in both subunits markedly impaired p51-p66 interaction, directly evidenced by a significant decrease in Vpr-p51^{W401A} mediated p66^{W401A} packaging. Based on our data, the structural analysis of several RT crystal structures and previous reports on the Trp-motif, we believe that repositioning the α L- β 20 loop by mutating p66^{W401} and disruption

of interactions involving p51^{W401}, p51^{W405}, p51^{N363} and p66^{W410} account for our findings. Our subunit-specific mutational analysis of the W401 RT mutants demonstrates that W401 of the p66 and p51 subunits has distinct structural roles in the stabilization of the RT heterodimer. In p51 the W401A mutation appears to affect the interactions at the interface, through disruption of the π - π interactions with p66^{W410}. However, the p66-W401A mutation appears to affect the folding of the p66 subunit because it is at the interface of two helices (α L and α K) (Figure 1C). Hence, when both subunits are mutated the different effects appear additive.

Our structural analysis of the dimer interface in several RT crystal structures highlighted the potential importance of a cluster of four tryptophans in p51 (W398, W402, W406 and W414) proximal to the dimer interface. While these four p51 tryptophans do not directly interact with p66 residues, they are clustered together through hydrophobic interactions and seem poised to indirectly affect the dimer interface by their proximity to residues Y405, W401, and N363 of p51 that are at the p51-p66 interface (Figures 1B and 1C). Our subunit-specific mutagenesis of these residues suggests that the Trp cluster affects the interaction between p51 and p66 (Figure 2). Interestingly, alanine substitution resulted in a misprocessed form of p66 that was detected in virions. *trans*-complementation analysis using PR defective virus indicates that a cellular protease is responsible for the aberrant processing of p66. We suggest two possible explanations for this observation. First, the p51 Trp mutants may interact with and incorporate into virions a smaller processed form of p66 generated in the cell. Our failure to detect the aberrant p66 in cell lysates, suggests it is present at a significantly lower level than wildtype p66. Alternatively, these p51 Trp mutants might form unstable heterodimers in which p66 is misfolded and thus, susceptible to proteolytic processing by a cellular protease. If this were true, it is interesting to

note that dimer instability causes p66 misprocessing instead of normal processing to generate p51, which could associate with disassociated p66 to give functional RT heterodimer. The defect in infectivity seen with the mutants containing misprocessed p66 further supports this interpretation. Although p66 misprocessing seems to occur as a consequence of the atypical manner in which RT is expressed via the *trans*-complementation approach, our results suggest that residues W398, W402, W406 and W414 are important for proper RT subunit interactions.

NNRTIs are a chemically diverse group of largely hydrophobic compounds that inhibit HIV-1 RT by binding in a hydrophobic pocket near the polymerase active site in the p66 subunit. NNRTIs have been described that can either stabilize or destabilize the RT heterodimer. Various NNRTIs have also been found to induce increased β -gal activity in the yeast two-hybrid system, due to enhanced RT subunit association ³⁰. In particular, efavirenz binding to the NNRTI hydrophobic pocket enhanced RT heterodimerization, including RT with p51/p66 W401 mutations. Additionally, both the 2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl 3'-spiro-5'-(4"-amino-1",2"-oxathiole 2",2"-dioxide) (TSAO) thymine derivatives and the *N*-acyl hydrazones are classes of compounds that show inhibition characteristics similar to NNRTIs. Although these drugs may not bind to the well-defined NNRTI binding pocket of HIV-1 RT, they bind to a region of RT close to and partially overlapping this site. Furthermore, in the presence of a denaturant like urea these compounds have been shown to destabilize HIV-1 RT heterodimerization ^{14; 45}. Our results show a dose-dependent enhancement of dimerization of the p51^{W401A}/p66^{W401A} RT mutant in the presence of efavirenz. Since our approach to analyzing subunit-specific structure/function is conducted in a context that is physiologically relevant to HIV-1 replication, it should provide valuable insight into the ability of small molecule inhibitors

to affect RT subunit interactions. Since RT is incorporated into virions as part of the Pr160^{Gag-Pol} precursor polyprotein, it may be argued that initial dimerization events may occur at the level of the polyprotein, making inhibitors that act at the level of p51-p66 interactions inconsequential at the level of Pr160^{Gag-Pol} dimerization. A recent study, albeit carried out in bacteria, suggests that EFV enhances proteolytic processing of a segment of Pr160^{Gag-Pol} ⁴⁶. Despite the need to carefully consider these factors, our system represents the first viable approach for analyzing the effects of RT dimerization inhibitors in the context of virus infectivity. With heightened interest in compounds that inhibit RT conformational flexibility as a novel drug design concept ²⁶, especially for drugs that will be effective against mutating RT targets, our results indicate the utility of this novel *trans*-complementation approach for assessing the effects of RT inhibitors on subunit interactions using infectious virions in a context that is more physiologically relevant.

Materials and Methods

Cells, antibodies and antiviral drugs

The 293T, JC53 ³², and TZM-bl cell lines ³³ were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). The JLTRG-R5 cell line ³⁴ was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 15% FBS and gentamycin (0.1 mg/ml). Antibodies used included polyclonal anti-RT and anti-Vpr sera ³⁵ and mAbs to human α -tubulin (Sigma), HIV-1 CA (183-H12-5C, contributed by Bruce Chesebro and Hardy Chen) and HIV-1 RT and RNase H (8C4 and 7E5), respectively, contributed by Dag E. Helland), obtained through

the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The NNRTI efavirenz (EFV) was obtained from the NIH AIDS Research and Reference Reagent Program.

Plasmid constructs

The HIV-1 pSG3 (SG3) proviral clone (Genbank Accession # L02317) ³⁶ was used to produce wildtype virus, and to construct the RT-deficient proviral clone and all recombinant RT and IN expression plasmids (for abbreviations of plasmids see Table I). The RT-IN-minus pSG3^{M7} (M7) proviral construct described previously ³¹ was used for *trans*-complementation analysis with all the pLR2P-based RT and IN expression plasmids. For expressing the RT subunits in *trans*, the pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) plasmid ³¹ was modified by including 135 bp of PR sequence 5' of RT. This increased the molecular weight of the Vpr-RT fusion protein and enabled visual separation from p66 by immunoblot analysis. Briefly, p51-encoding sequence was PCR amplified from SG3 using primers containing BglII and MluI restriction sites, respectively. The internal ribosome entry site (IRES) was PCR amplified from the encephalomyocarditis virus (EMCV) (Genbank Accession # NC_001479) with primers containing MluI and XmaI sites, respectively. The p51 and IRES DNA fragments were digested with corresponding endonucleases and ligated simultaneously into the BglII-XmaI cut pLR2P-vprRT ³⁷. The *vpr-p51/p66* was modified in that the N-terminal protease cleavage (PC) site of RT was maintained by including 135 bps of PR-encoding sequence 5' of RT compared to 33 bps of PR sequence in the original construct. The *vpr* and p51 coding sequences were placed in-frame, with a translational stop codon (TAA) to terminate RT expression at amino acids 440,

which is the full-length p51 subunit. The *vpr-p51* reading frame was followed by the IRES and then the p66-encoding DNA sequence. Mutant derivatives of *vpr-p51/p66* (Table I) were constructed using PCR-based site-directed mutagenesis and cloning into the BglII-MluI or XmaI-XhoI sites for p51- and p66-containing DNA fragments, respectively. The pLR2P-*vpr-Δp51*-IRES-p66 (*vpr-Δp51/p66*) control expression plasmid was constructed to contain a translational stop codon at the first amino acid position of p51³¹. This plasmid controls for non-specific incorporation of p66 into viral particles. All mutant clones were confirmed by nucleotide sequence analysis. The pLR2P-*vprIN* (*vpr-IN*) expression vector has been described previously

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Transfection and analysis of virus infectivity

DNA transfections were performed on monolayer cultures of 293T cells grown in 6-well plates using the calcium phosphate DNA precipitation method. Unless otherwise noted, each cell monolayer (well) was transfected with 6 µg of proviral DNA, 3 µg of the *vpr-p51/p66* constructs and 1 µg of the *vpr-IN* construct. Culture supernatants from the 293T cells were collected 60 h post-transfection, clarified by low-speed centrifugation (1000 x g, 10 min), and filtered through 0.45 µm pore-size sterile filters. The clarified supernatants were analyzed for HIV-1 p24 concentration by ELISA (Beckman-Coulter Inc.).

Virus infectivity was assessed using the TZM-bl reporter cell line as described earlier³³. Briefly, virus containing supernatants were normalized for p24 antigen concentration, serially diluted (five-fold dilutions) and used to infect monolayer cultures of TZM-bl cells. At 48 h post-

infection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) reagent as described³⁸. Following X-gal staining, the blue-stained cells were counted using a light microscope. Wells containing between 30 and 300 blue cells were used to calculate the infectious units of virus per ng of p24 antigen (IU/p24-ng).

The ability of *trans*-RT-containing virions to infect T cells was tested by quantitatively analyzing infection of the JLTRG-R5 reporter T cell line. 12-well flat-bottomed culture plates containing 1.0×10^5 JLTRG-R5 cells were infected at a multiplicity of infection (MOI) of 5.0 (as determined by the TZM-bl assay) for the wildtype Vpr-p51/p66 complemented virions. The other *trans*-RT-containing virion preparations were normalized for p24 antigen concentration equivalent to that of the wildtype *trans*-RT. The total volume was adjusted to 1 ml and the infection was carried at 37°C for 24 h. At 24 h post-infection, 1 ml of fresh RPMI 1640 was added to each well and culture was continued at 37°C for an additional 48 h. Then the cells were washed (2x) in phosphate buffered saline (PBS). The cell pellet was resuspended in 50 μ l PBS and then fixed in 1% paraformaldehyde (in PBS). The expression of EGFP was measured using a FACStar Plus flow cytometer with CellQuest software (Becton Dickinson).

Effects of NNRTIs on *trans*-RT subunit interaction

DNA transfections were performed on monolayer cultures of 293T cells grown in 6-well plates using the FuGENE 6 Transfection Reagent (Roche), as recommended by manufacturer. The M7 and *vpr-p51/p66*-based plasmids were used at a ratio of 2:1. At 24 h post-transfection, the specified concentrations of drug were added. Culture supernatants from the 293T cells were collected 60 h post-transfection, clarified by low-speed centrifugation (1000 x g, 10 min), and

filtered through 0.45 μ m pore-size sterile filters. The clarified supernatants were processed for and analyzed by immunoblot as described below.

Immunoblot analysis

Transfection-derived virions were concentrated by ultracentrifugation through 20% sucrose cushion (125,000 x g, 2 hr, 4°C) using a SW41 rotor (Beckman Inc.). Pellets were solubilized in Laemmli loading buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol), boiled, and proteins were separated on 12.0% polyacrylamide gels containing SDS. Following electrophoresis, proteins were transferred to nitrocellulose (0.2- μ m pore size) by electroblotting and incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in PBS). The blocked blots were exposed to an appropriate primary antibody for 1 h in blocking buffer with constant mixing. After extensive washing, bound antibodies were detected by chemiluminescence using horseradish peroxidase-conjugated species-specific secondary antibodies (Southern Biotechnology Associates, Inc.).

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Figure legends

Figure 1. Structural analysis of RT connection subdomain. (A) Alignment of Trp-motifs of primate lentiviruses. The Pol amino acid sequences of representative strains of primate lentiviruses were aligned using MegAlign (DNASTAR, Inc.). HIV-1 RT sequence (amino acids 395-415) is shown along with corresponding alignments for other indicated primate lentiviruses. (B) Ribbon representation of the p66 (cyan) and p51 (yellow) subunits in the crystal structure of the complex of HIV-1 RT with double-stranded DNA and incoming tenofovir-diphosphate (pdb file 1T05)⁴⁷. For clarity, only the protein is shown. The tryptophan-rich motif and other p51 residues at the interface of the two subunits are shown in Van der Waals volumes (red). Residues W401 and W410 of the p66 subunit are shown at or near the interface also in Van der Waals volumes (magenta). Residue W401 (red) of the p66 subunit and residue N363 (magenta) of the p51 subunit are shown at interacting distance at the subunit interface. (C) Magnification of the area in the box shown in "B". Shown are the side-chains of residues of the tryptophan motif and

of the interface that were mutated in this study. (D) Ribbon representation of the interface between p66 and p51. W410 of the p66 subunit is shown to have extensive interactions with residues of the p51 subunit (p51-N363, p51-W401, and p51-Y405).

Figure 2. Analysis of p51 Trp-motif mutants. M7 proviral DNA was transfected into 293T cells alone or together with wildtype or mutant *vpr-p51/p66* and *vpr-IN* expression plasmid DNAs. Transfection-derived virions were analyzed by immunoblotting for (A) RT (p51/p66) and (B) CA (p24). Expression of Vpr-p51 (C), p66 (D) and α -tubulin (E) in the transfected 293T was examined by immunoblotting. (F) Infectivity of p51 Trp-motif mutants. The infectivity of virions containing alanine substitutions in the p51 Trp-motif was analyzed using the TZM-bl reporter cell line as described in Materials and Methods. Infectivity is expressed as a percentage of the wildtype *trans*-RT heterodimer (Vpr-p51/p66) complemented virions.

Figure 3. Analysis of Trp-motif residues located at the RT heterodimer interface. Trp-motif residues that lie within interacting distance at the dimer interface were mutated. The infectivity of virions containing single (A) or dual (B) mutations was analyzed by the TZM-bl reporter cell assay. Infectivity is expressed as a percentage of the wildtype *trans* heterodimer control.

Figure 4. Analysis of W401 and W410 mutations in proviral DNA. (A) The importance of RT Trp-motif residues W401 and W410 for viral infectivity was analyzed using the HIV-1 NL4-3 molecular clone. Infectivity was determined using TZM-bl reporter cells and the results

are expressed as a percentage of wildtype NL4-3. Virions derived by transfection of the wildtype and mutant proviral DNAs were also analyzed by immunoblotting using mAbs to RT (B) and CA (C).

Figure 5. Subunit specific analysis of the W401A mutant. The W401 residue was mutated in p51, p66 or p51 and p66. Transfection derived virions containing the respective mutant *trans* RTs, were analyzed for (A) infectivity on TZM-bl cells and (B) virion incorporation of p51 and p66 by immunoblotting. (C) Virion infectivity was determined using the TZM-bl reporter cells (black bars) and the JLTRG-R5 reporter T cell line (white bars). Infectivity is expressed as a percentage of the wildtype *trans*-RT control.

Figure 6. Effect of NNRTIs on RT subunit interactions. Virions were generated by cotransfection of 293T cells with M7 and *trans*-RT dimerization-defective mutant plasmid vpr-p51^{W401A}/p66^{W401A}. The dimerization enhancing NNRTI EFV was added to the culture medium 12 h after DNA transfection at concentrations ranging from 0.01-1.0 μ M. The transfection-derived virions were collected 48 h later and analyzed by immunoblot using mAbs to (A) RNase H and (B) CA.

Table

Table 1. Abbreviations for plasmids

Plasmid	Abbreviation
pSG3 ^{wt}	SG3
pSG3 ^{M7}	M7
pLR2P-vpr-p51-IRES-p66	vpr-p51/p66
pLR2P-vpr-Δp51-IRES-p66	vpr-Δp51/p66
pLR2P-vpr-p51-IRES-p66 ^{MUTANT}	vpr-p51/p66 ^{MUTANT}
pLR2P-vpr-p51 ^{MUTANT} -IRES-p66	vpr-p51 ^{MUTANT} /p66
pLR2P-vpr-IN	vpr-IN

The abbreviations for plasmids used in this study are listed above. All mutants were abbreviated in a similar style as shown for ^{MUTANT}.

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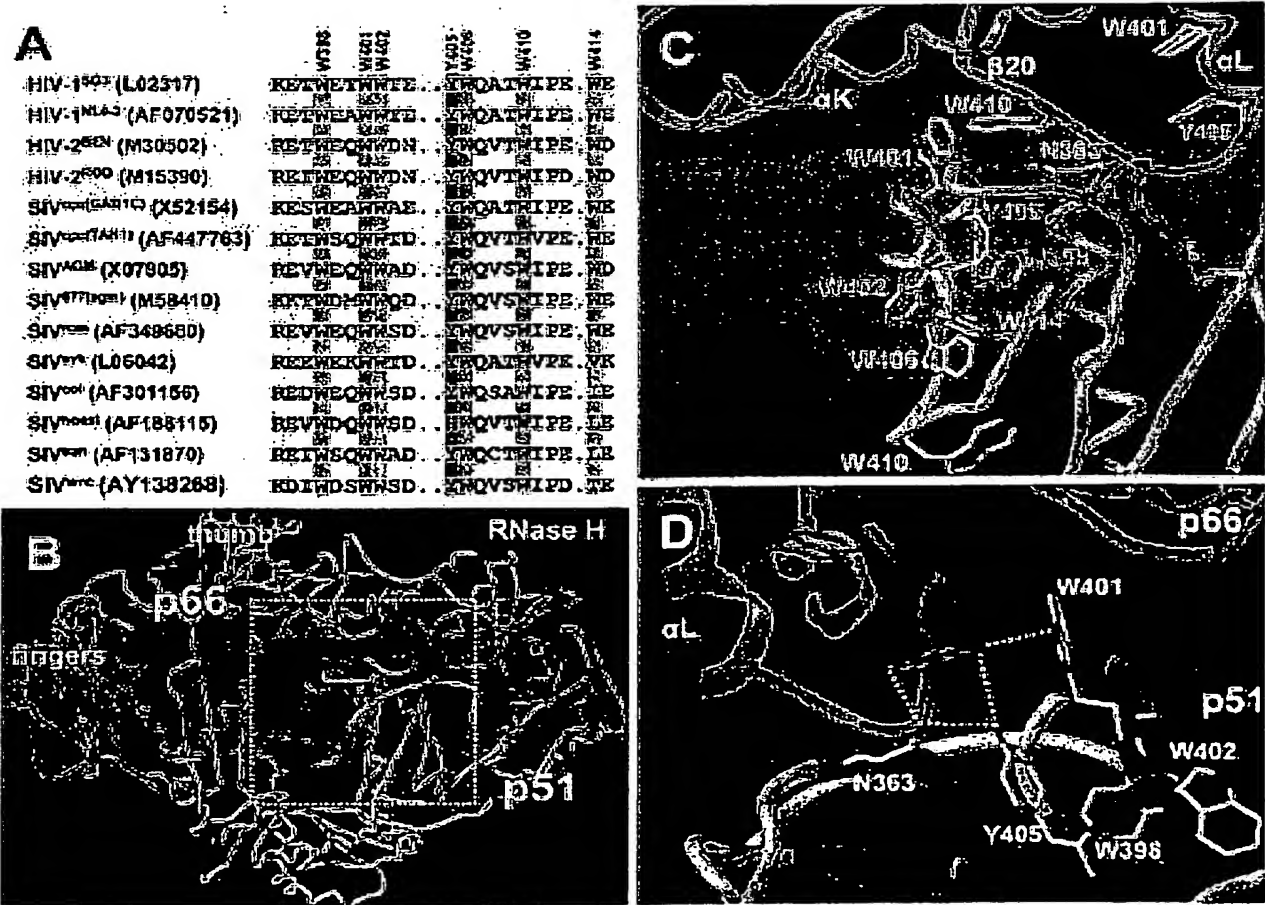


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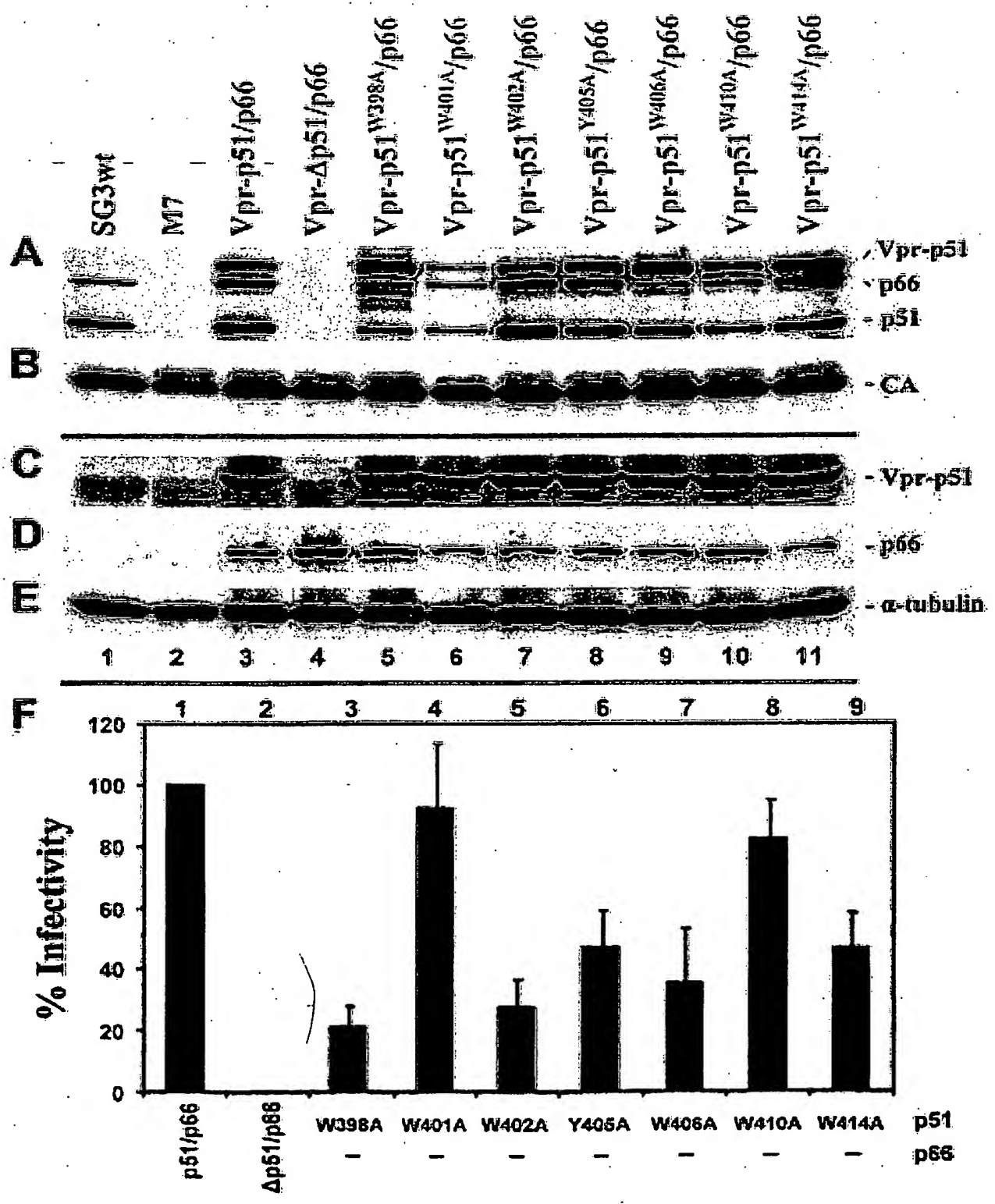


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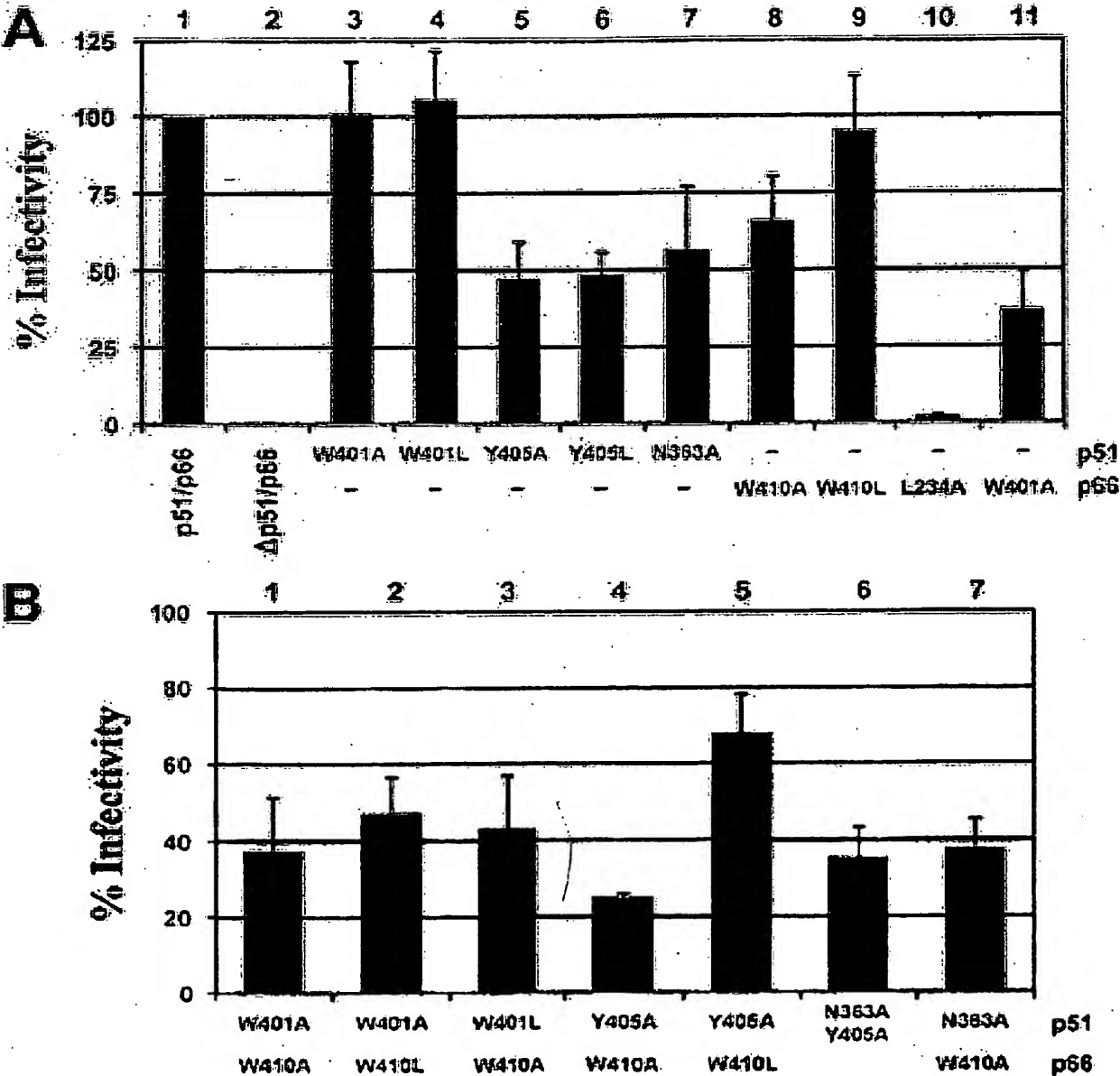


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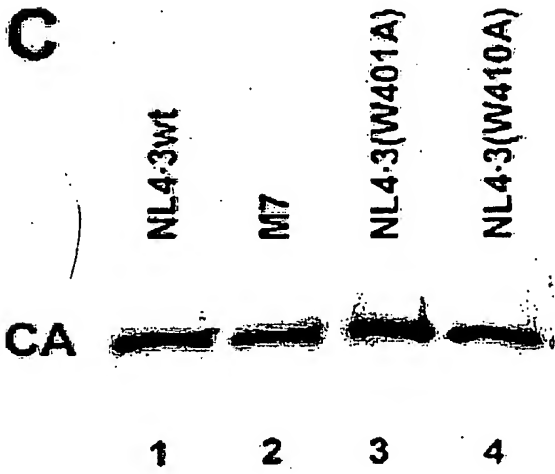
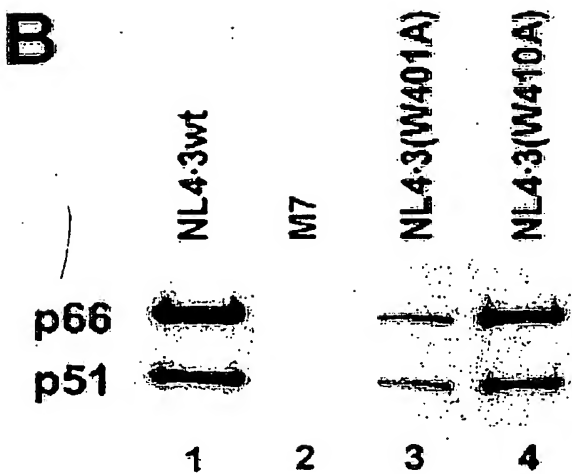
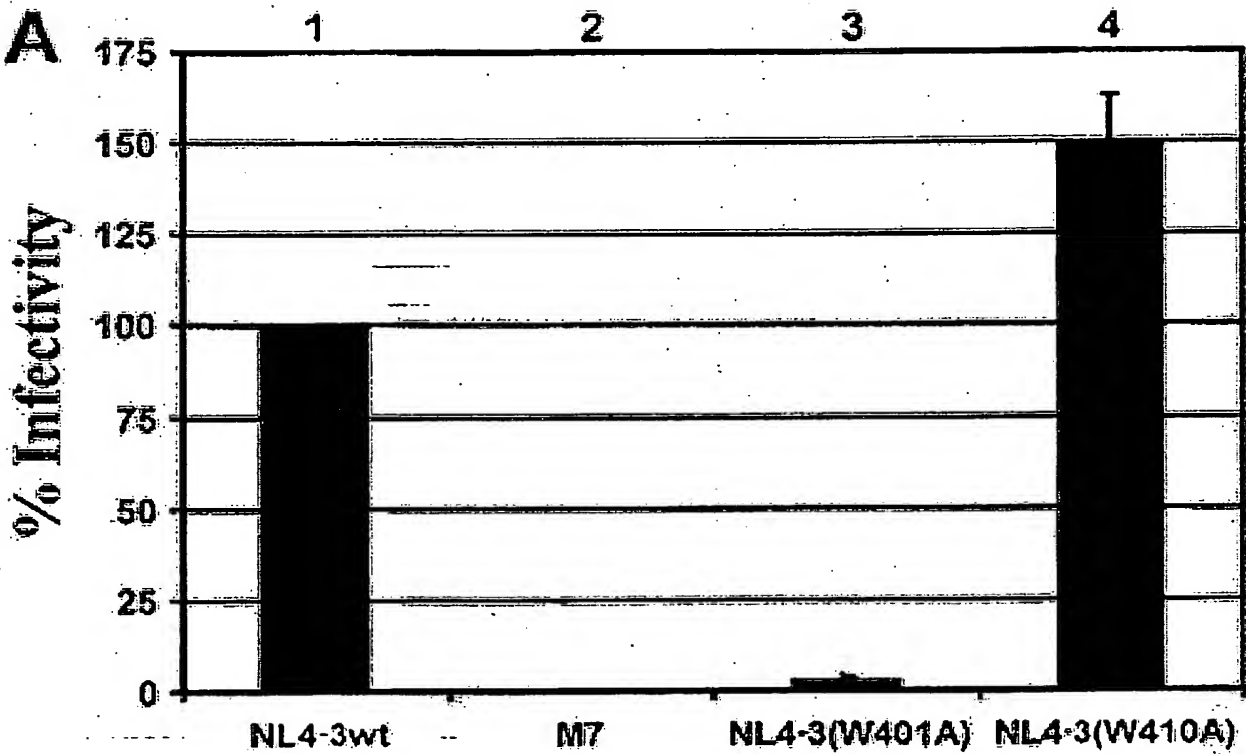


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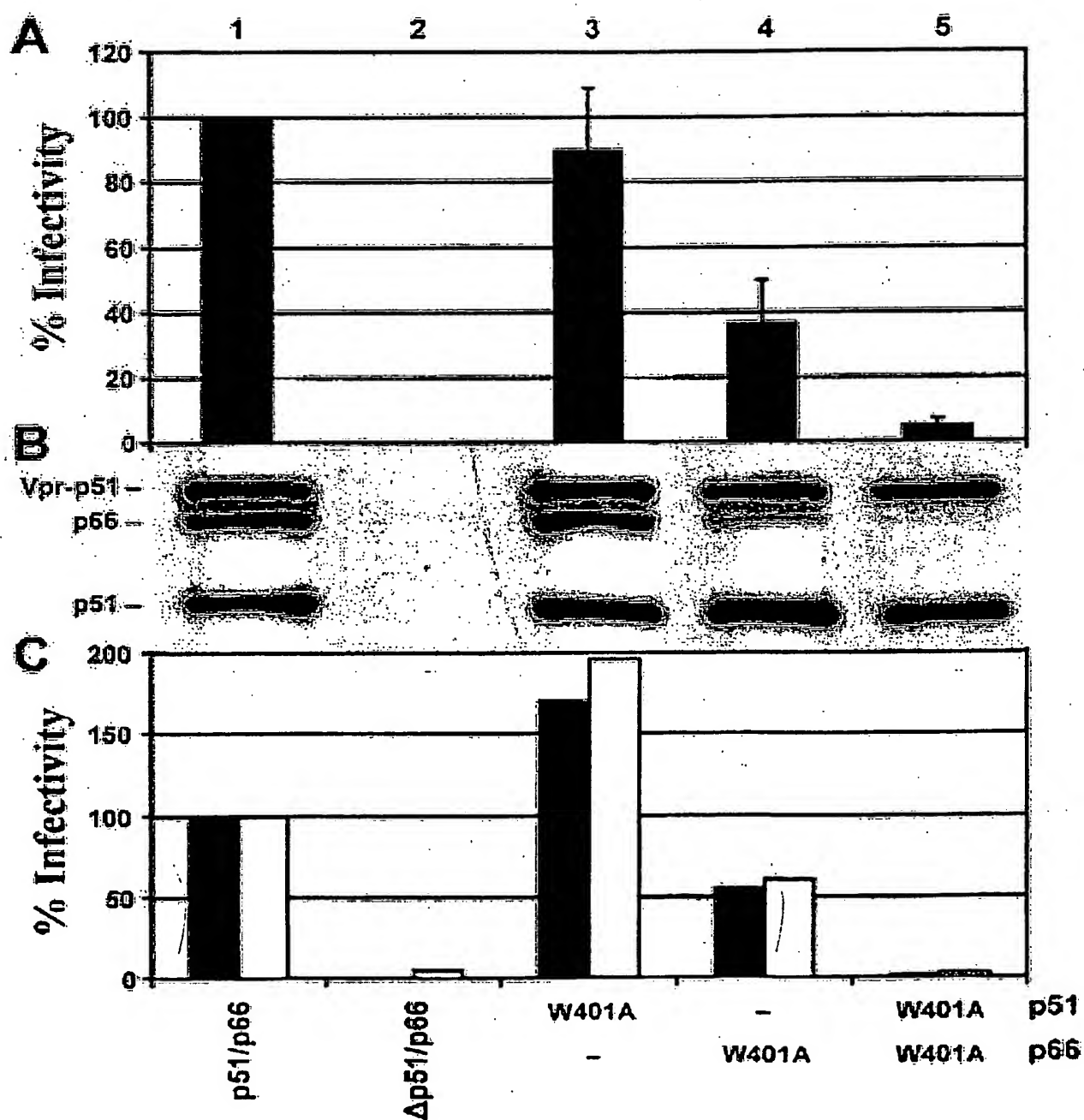
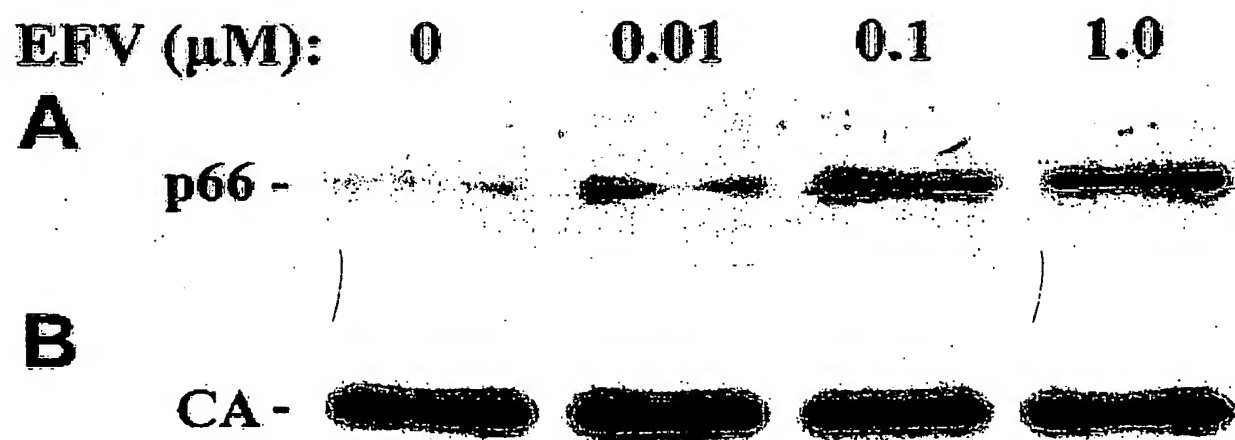


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